

EVALUATION OF THE ANTI-INFLAMMATORY ACTIVITY OF THE LINIMENT FROM THE LEAF EXTRACT OF *VITEXNEGUNDO* FAMILY *VERBENACEAE*

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Abstract

This study was conducted to evaluate the anti-inflammatory activity of the liniment from the leaf extract of *Vitexnegundo* family *Verbenaceae*. The leaves were extracted using Soxhlet apparatus. In-vitro protein stabilization test were done using the different concentrations of the leaf extract, 100%, 75%, 50%, and 25% and were used to treat the proteins in the albumin solution. The twenty five percent (25%) concentration exhibited the greatest anti-inflammatory reading based on the highest transmittance, a liniment was formulated using alcohol and oil as a base. The alcohol-based and oil-based liniments were tested using spectrophotometric analysis to determine if there was any significant difference on their anti-inflammatory action. Sensitivity test was also done using Patch Test and Scratch Test. After performing all the tests, the researchers concluded that the leaf extract has the ability to stabilize albumin and delay the onset of visible signs of denaturation making it less turbid and therefore increase transmittance reading, which indicates its effectiveness as a potential anti-inflammatory agent.

Keywords: Vitexnegundo; Liniment; Anti-inflammatory activity; Herbal medicine; Medicinal plants; Leaf extract

Introduction

Health is an imperative aspect of human beings. It is understood as an implication of socio-economic and political conditions. Nevertheless, each individual is responsible for upholding and restoring their own health more

so if it is at stake. There are many ways to prevent and cure conditions that diminish the quality of life of an individual. However, most Filipinos cannot cope with the increasing cost of medical treatment. This can be attributed to that fact that our country has patterned its health care system to Western Medicine, so it has become very expensive considering that they are using technological equipments, techniques and high quality materials. Due to this fact, an individual needs to seek alternatives to solve health problems. (Cruz & Jubilo, 2014; Cruz, Alcantara & Cruz, 2014) This is why traditional herbal medicine is becoming a trend nowadays. Plants are the oldest healing materials known to man and one of which is *Vitexnegundo*.

Preparations of *Vitexnegundo* have been used for a wide variety of complaints traditionally, although scientific research has concentrated on its use for respiratory complaints. *Vitexnegundo* is generally accepted in the Philippines to be useful for coughs, asthma symptoms, and other respiratory problems, and the Philippine government actively promotes it as an alternative to Western cough medicines. Some doctors also prescribe *Vitexnegundo* to assist in the treatment of asthma, as regular doses appear to reduce the strength of asthma attacks. (Arcangelo, 2001) In the study of Dharmasiri, Jayakody, Galhena, Liyanage, and Ratnasooriya, (2003), they confirmed the oral anti-inflammatory, analgesic and antihistamine properties of mature fresh leaves (MFL) of *Vitexnegundo* L. (Verbenaceae) claimed in the Ayurveda medicine by orally treating a water extract of the leaves to rats. Another study showed that *Vitexnegundo* also possesses anti-inflammatory activity which was more pronounced on subacute rather than on acute inflammation. The analgesic and anti-inflammatory action of *Vitexnegundo* can be attributed to its flavonoid contents, which are known to act through inhibition of prostaglandin biosynthesis. (Telang, Chatterjee, and Varshneya, 1999)

According to Howard C. Ansel, liniments are alcoholic or oleaginous solutions or emulsions of various medicinal substances intended to be rubbed on the skin with friction. Liniments are used for various therapeutic effects depending on the ingredient they contain. (Ansel, Allen, Popovich, 2005). Given that *Vitexnegundo* has a potential anti-inflammatory property, the researchers chose to incorporate the *Vitexnegundo* extract in the liniment using both alcohol and oil as a base.

Inflammation is the complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells, or irritants. It is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, and various cells within the injured tissue (Kee, 1997).

In these situations, there are different options to alleviate pain, depending on the type of inflammation and tissue involved. They can be treated with steroidal or non-steroidal medications. These medications can be applied topically, taken orally, injected intravenously or inhaled either through the nose or mouth. They are available as over-the-counter or prescription drugs. In addition, because of the production cost, these drugs are expensive and sometimes not affordable to the common masses. Due to this fact, poor communities prefer the traditional method of treating an inflammation using herbs.

This study was conducted to primarily confirm potential anti-inflammatory property of the *Vitexnegundo* leaf extract. The extract was tested using the in-vitro protein stabilization test to confirm its anti-inflammatory potential. The researchers aimed to formulate a new dosage form using *Vitexnegundo* leaf extract as a topical anti-inflammatory. Since syrups and tablets used as cough remedies are already available in the market, the researchers decided to formulate a liniment. Moreover, a liniment was formulated and experimented to determine its potential anti-inflammatory activity, and a sensitivity test for local and topical applications was also done.

The study would benefit the society because it would lessen the cost of using commercially prepared drugs for the alleviation of pain. In addition, the researchers would like to contribute to the promotion of health through cost effective herbal products. The study serves as a milestone in order to know other benefits of *Vitexnegundo* plant to help in the promotion of health of the common masses. Furthermore, the study would enhance our knowledge regarding drugs and their mechanism of action and to utilize the knowledge to discover other products that would be useful to the society.

Objective of the Study

This study dealt with the evaluation of the anti-inflammatory activity of the liniment from the leaf extract of *Vitexnegundo* family *Verbenaceae*. It specifically aimed:

1. Determine the most effective concentration of *Vitexnegundo* leaf extract as anti-inflammatory using the in-vitro protein stabilization test;
2. Determine the difference in anti-inflammatory activity of an alcohol-based liniment and an oil-based liniment containing the 25% concentration of the leaf extract and when compared with Methylsalicylate using the in-vitro protein stabilization test and the sensitivity test;

3. Evaluate the safety and effectiveness of the liniments based on the in-vitro protein stabilization test and the sensitivity tests for local and topical applications.

Materials and Method

This study employed the descriptive and experimental method of research for the evaluation of the anti-inflammatory activity of the liniment from the *Vitexnegundo* leaf extract.

Pure extract, and three different concentrations, 25%, 50% and 75% of the extract were prepared. A liniment was formulated using two vehicles, oil and alcohol. The different concentrations were subjected to in-vitro protein stabilization test to determine the most effective concentration. The most effective concentration was then formulated as a liniment, using two different bases which were oil and alcohol. The two liniments with the different bases were evaluated through the in-vitro protein stabilization test and the sensitivity test. The liniment which had the better anti-inflammatory activity and which exhibited a lower primary irritation index was then compared to Methylsalicylate liniment, using the in-vitro protein stabilization test and the sensitivity test. The results obtained from the sensitivity test were evaluated descriptively.

Extraction of the Sample using the Soxhlet Apparatus

Fifty grams of the *Vitexnegundo* leaves was placed in a porous paper extraction thimble which is then fitted into the extraction tube. Methanol was used as a solvent. A reflux condenser was attached to the extraction tube. The solvent vapors passed upward from the flask and through the side tube of the extractor to the condenser. The condensate dripped into the thimble until a sufficient amount has accumulated to raise the level of liquid in the extraction tube to the top of the siphon tube. The solution that stood in contact with the sample was then discharged into the flask where the solvent was vaporized again. The solvent passed through this cycle repeatedly. The extract was collected in the flask and was prepared to the different concentrations (75%, 50%, 25%) for spectrophotometric analysis (Knevel and Digangi, 1977).

In-Vitro Protein Stabilization Test

Preparation of the Different Concentrations of the *Vitexnegundo* leaf extract

Ten milliliters of the different concentrations were prepared by dissolving the *Vitexnegundo* leaf extract in distilled water. To prepare the 75%, 50%, 25% concentrations, 7.5mL, 5mL, and 2.5 mL of the leaf extract were dissolved respectively in a sufficient amount of distilled water to make

10mL. One percent weight-in-volume albumin solution was added to each of the flask to bring the solutions to a final volume of 15 mL. Each solution was mixed thoroughly.

Preparation of the Albumin Solution

One (1) mL of egg white is dissolved in one hundred (100) mL of distilled water.

Preparation of the Reference Standard

Fifty (50) mL of the positive control, Methylsalicylate was accurately weighed. The sample was transferred to a one hundred (100) mL beaker with forty two (42) mL of distilled water and stirred, five (5) mL of 1% Polysorbate 80 and stirred. The 1% Polysorbate 80 was used to solubilize the Methylsalicylate with distilled water. The solution was transferred to a volumetric flask and diluted to one hundred (100) mL by further addition of 1% weight-in-volume albumin solution. This solution was designated as the stock solution.

Spectrophotometric Analysis

The prepared samples of the different concentrations and the reference standard were placed in separate test tubes and warmed using a water bath at 70°C in order to produce sufficient thermal denaturation of proteins. Turbidity was then produced and was measured using the spectrophotometer at 460nm. Transmittance readings were recorded and results were compared with the reference standard. A higher transmittance reading implies a greater anti-inflammatory activity.

Preparation of Lagundi Liniment

1. Oil-Based *Vitexnegundo* Liniment.Eighty (80) grams of cotton seed oil was placed on a suitable dry flask or bottle, on a steam bath. Twenty (20) grams of *Vitexnegundo* extract was added and the container was stoppered securely. After which, the solution was agitated to dissolve the *Vitexnegundo* without further heating. (Hernandez, Dayco, 1994)

2. Alcohol-Based *Vitexnegundo* Liniment.To prepare the alcohol-based liniment, approximately five grams of the *Vitexnegundo* extract and one gram of Rosemary oil was dissolved in seventy milliliters of alcohol. Six grams of dried and granulated soap was added with sufficient quantity of purified water to measure 100 ml. The mixture was agitated to dissolve the soap. The solution was kept for twenty four hours and was filtered. (Hernandez, Dayco, 1994)

F. Protein Stabilization Test of Lagundi liniment

Fifty (50) mL of the *Vitexnegundo* liniment was accurately weighed. The sample was transferred to a one hundred (100) mL beaker with forty two (42) mL of distilled water and stirred; five (5) mL of 1% Polysorbate 80 was added and stirred. The 1% Polysorbate 80 was used to solubilize the *Vitexnegundo* liniment with distilled water. The solution was transferred to a volumetric flask and diluted to one hundred (100) mL by further addition of 1% weight-in-volume albumin solution. This solution was warmed using a water bath at 70°C to produce sufficient turbidity and was analyzed using the spectrophotometer. Transmittance readings were recorded and compared to a positive control.

Sensitivity for Local and Topical Application

A. Patch Test. A group of four (4) male guinea pigs were selected. The skin lateral to the spinal groove was shaved and cleaned. The left side of the groove in the animal was utilized as the negative control site and the right side as the test drug site. The sites were cleaned with 70% alcohol. The test drug (*Vitexnegundo* leaf extract) and the negative control drug (cotton seed oil) were delivered on the inoculation sites respectively. Both sites were covered with sterilized gauze (1x1 cm in size). Surgical tapes were used to keep it in place. It was left undisturbed for 24 to 72 hours during which time all the test animals are rendered immobile. The patches were removed after 24 hours of exposure and the reactions were evaluated according to scores. The patches were returned and another scoring was done after 72 hours. The average scores of the 24 and 72 hours reading was computed. The average of the scores for the patch and scratch tests were combined. This combined average was referred to as the primary irritation index.

B. Scratch Test. The procedure in scoring method for this test was similar as to the patch test but with slight modifications. The same numbers of test animals were used as in the patch test. The skin was abraded, lateral to the spinal groove of the test animal, by slightly scratching the skin five to seven times with a 20 gauge hypodermic needle. The test drug was applied on the right side of the abraded skin and the left side was applied with the control. The results were observed and recorded.

Population of the Study

Twelve (12) guinea pigs of 250-300 grams of body weight were used as the test animals for this study. A single gender (male) was used to obtain a more consistent data limiting the occurrence of variations and to attain more established conclusions. The guinea pigs were subjected to sensitivity test for local and topical application to evaluate the safety and efficacy of the formulated *Vitexnegundo* leaf extract liniment.

Treatment of Data

The most effective concentration was determined by the ability of the sample extract to stabilize proteins which was shown on how much protein was denaturated. Protein denaturation was measured using comparison of the transmittance readings. Transmittance measures the light that passes through the substance. The results gathered from the in-vitro test were analyzed using statistical method. This includes f-test analysis of variance (ANOVA). The concentration with the highest transmittance reading was considered as the most effective.

Analysis of variance (ANOVA) is essential in this study to determine if there is a significant difference in them anti-inflammatory effect of the different concentrations of the *Vitexnegundo* leaf extract with that of the positive control, Methylsalicylate. The F-value was computed from the data and compared with the critical F-value. Any increase in the T-value, means that more than the critical value may mean a significant difference in the anti-inflammatory activity of the samples. This was indicated by the difference in their transmittance readings. The higher the transmittance reading, the greater was the anti-inflammatory activity of the sample.

The results obtained from the sensitivity test were analyzed descriptively. Based on the scores gathered, a primary irritation index was computed. The results obtained implied that the alcoholic and oil-based liniment are non-irritant thus, safe and effective as an anti-inflammatory agent.

Results

In-vitro Protein Stabilization Test (*Vitexnegundo* Extract)

Table 1 presents the transmittance readings obtained from the *Vitexnegundo* leaf extract in different concentrations (100%, 75%, 50%, and 25%). The In-vitro Protein Stabilization Test reveals the anti-inflammatory activity of the 25%, 50%, 75% and 100% leaf extract of *Vitexnegundo*. 25% has a mean transmittance of 71.5, 50% has 58.0, 75% has 55.5 and 100% has 53.5. This shows that the 25% leaf extract has the highest anti-inflammatory activity compared to the other concentrations.

Table 1. Protein Stabilization Test employing Spectrophotometric analysis (*Vitexnegundo* Extract)

| Concentration | Transmittance Trial 1 | Transmittance Trial 2 | Mean |
|---------------|--------------------------|--------------------------|------|
| 100% | 54 | 53 | 53.5 |
| 75% | 55 | 56 | 55.5 |
| 50% | 59 | 57 | 58 |
| 25% | 72 | 71 | 71.5 |

Test of Significance Using the Analysis of Variance (ANOVA)

Table 1a presents the statistical treatment to determine if there are significant differences in the anti-inflammatory activity of the different concentrations of the *Vitexnegundo* leaf extracts based on the transmittance readings.

From the table, the computed F-value of 151.00 is much greater than the tabular value of 6.59, implying significant differences. This means that there are varying levels of anti-inflammatory activity of the different concentrations of the *Vitexnegundo* leaf extracts. To determine where the differences happen, a follow-up test was done.

Table 1a. Test of Significance of Transmittance Readings at Different Concentrations Using the Analysis of Variance (ANOVA)

| Sources of Variations | Sums of Squares | Df | Mean Square | F-Value | Tabular Value | Interpretation |
|-----------------------|-----------------|----|-------------|---------|---------------|----------------|
| Between | 396.375 | 3 | 132.125 | 151.00 | 6.59 | Significant |
| Within | 3.50 | 4 | 0.875 | | | |
| Total | 399.875 | | | | | |

Multiple Comparisons Using Tukey Procedure:

Table 6B is the follow-up test for ANOVA since it shows a significant difference in the test. Multiple Comparisons using Tukey procedure was done as a follow-up test to determine where the differences happen.

Table 1b. Multiple Comparisons of different concentration Using Tukey Procedure

| COMPARISONS | Mean Difference | HSD Value | Interpretation |
|-----------------------------|-----------------|-----------|-----------------|
| 25% and 50% concentrations | 13.50 | 3.810 | Significant |
| 25% and 75% concentrations | 16.00 | 3.810 | Significant |
| 25% and 100% concentrations | 18.00 | 3.810 | Significant |
| 50% and 75% concentrations | 2.50 | 3.810 | Not Significant |
| 50% and 100% concentrations | 4.50 | 3.810 | Significant |
| 75% and 100% concentrations | 2.00 | 3.810 | Not Significant |

A comparison of the 25% leaf extract to the 50%, 75% and 100% extracts showed mean differences that are all higher than the HDS value indicating significant differences. This means that the 25% leaf extract has the best anti-inflammatory activity among the different concentrations. Similarly, a comparison of the 50% to the 100% extract showed a mean difference that is higher than the HSD value showing that the former concentration has a better anti-inflammatory activity than the latter concentration. On the other hand, a comparison of the 50% to the 75% extract and the 75% to the 100% extract, the mean differences are lesser than the HDS value implying significant differences. This means that the 50% and

75% leaf extracts have similar anti-inflammatory activities while the 75% and 100% extracts have similar anti-inflammatory activities.

Assuming that *Vitexnegundo* is a potent drug, twenty five percent concentration already exhibits an anti-inflammatory activity and higher concentrations may render it toxic.

In- Vitro Protein Stabilization Test (Vitexnegundo Liniment)

Table 2 presents the transmittance readings obtained from the *Vitexnegundo* liniment which contains 25% *Vitexnegundo* leaf extract prepared into two bases, oil and alcohol. The oil-based liniment exhibited a mean transmittance of 14 while the alcohol based liniment exhibited 13 mean of transmittance. The control showed a mean transmittance of 12. This shows that the oil-based formulated liniment has greater anti-inflammatory activity compared to the alcohol-based and to the control.

Table 2. In-Vitro Protein Stabilization Test of L *Vitexnegundo* Liniments employing Spectrophotometric analysis

| | Transmittance Trial 1 | Transmittance Trial 2 | Average |
|--|--------------------------|--------------------------|---------|
| Alcohol Based Liniment of the 25 % leaf extract | 15 | 11 | 13 |
| Oil Based Liniment of the 25% leaf extract | 13 | 15 | 14 |
| Methysalicylate (Efficascent Oil) | 10 | 14 | 12 |

Test of Significance Using the Analysis of Variance (ANOVA)

The table 2a shows the results of the ANOVA procedure done to determine if there are no significant differences in the anti-inflammatory activity of the two liniment formulations and the positive control based on their transmittance readings. The table shows a computed F-value lesser than the tabular value ($0.333 < 9.55$), implying no significant differences. This further means that formulated liniments and the positive control have similar anti-inflammatory activities.

Table 2a. Test of Significance between the Lagundi liniments (Alcohol-based and oil- bases) and positive control Using the Analysis of Variance (ANOVA)

| Sources of Variations | Sums of Squares | Df | Mean Square | F- Value | Tabular Value | Interpretation |
|--------------------------|--------------------|----|----------------|-------------|------------------|-----------------|
| Between | 4.00 | 2 | 2.00 | 0.333 | 9.55 | Not Significant |
| Within | 18.00 | 3 | 6.00 | | | |
| Total | 22.00 | | | | | |

Sensitivity for Local and Topical

The oil-based liniment registered an average of 0.5 and 1 in the patch test and scratch test respectively after 24 hours of topical application.

Further, no signs of skin irritation were observed after 72 hours of application. On the other hand, the alcohol-based liniment registered a mean of 2 for both the scratch and patch test after 24 hours. After 72 hours, it registered a 0.5 mean for both test.

Discussion

In-vitro Protein Stabilization Test (*Vitexnegundo* Extract)

Based from the result, the 25% concentration had the highest transmittance reading which implies that it has the greatest anti-inflammatory activity. This may be attributed to the dose-response theory that states that a specific dose elicits a therapeutic effect and higher doses may render the drug toxic. Since 25% concentration already exhibits anti-inflammatory activity, an increase in concentration may lead to greater anti-inflammatory activity and eventually lead to toxic effects. As the concentration increases, the transmittance decreases implying a lesser anti-inflammatory activity on higher concentrations. Assuming that *Vitexnegundo* is a potent drug, 25% percent concentrations already exhibits an anti-inflammatory activity and higher concentrations may render it toxic.

In addition, experimental studies using various animal models have demonstrated that different parts of the plant especially leaves, fruits, roots and seeds possess anti-inflammatory activity (Jana, Chattopadhyay & Prasad, 1999). The anti-inflammatory activity may be due to prostaglandin synthesis inhibition, antihistamine, membrane stabilising and antioxidant activities (Dharmasiri, Jayakody, Galhena, Liyanage, & Ratnasooriya, 2003).

In- Vitro Protein Stabilization Test (*Vitexnegundo* Liniment)

Based on the results, the transmittance reading of the two formulated liniments (Oil-based and alcohol-based with the 25% leaf extract) is near the value of the transmittance reading for the positive control, implying that they have almost the same anti-inflammatory activity based on protein stabilization. The anti-inflammatory activity of the two liniment preparations are as effective as the commercially prepared liniment used as the control. This means that the 25% extract liniments can be used as substitute anti-inflammatory liniment.

Sensitivity for Local and Topical

Both the oil-based and alcohol based liniment showed only negligible skin redness and elevations which implies that it is non-irritant. However, the oil-based liniment showed the least sign of skin irritation than the alcohol liniment.

Conclusion

Based on the results obtained, the researchers conclude that:

1. At twenty five percent concentration, the *Vitexnegundo* leaf extract is more effective as an anti-inflammatory than other higher concentrations.
2. The lower the concentration of the extract, the higher transmittance reading and the greater anti-inflammatory activity.
3. There is no significant difference between an alcohol-based liniment and an oil-based liniment. The lower irritation index and the higher transmittance reading implied greater anti-inflammatory activity.
4. The oil-based liniment is safe, effective, and non-irritant as implied by the result from the sensitivity test for local and topical application using Patch Test and Scratch Test.

Recommendations

For further studies, the researches recommend the following:

1. Formulation of other dosage forms aside from liniments as an anti-inflammatory agent.
2. Test for the anti-inflammatory property of other Verbenaceae specie found in the country.
3. Test for the anti-inflammatory activity using other concentrations and using other dosage forms.
4. To test other potential activity of Lagundi aside from anti-inflammatory and cough and cold agent.

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